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1 **Genome and Plasmid Analysis of *bla*_{IMP-4}-Carrying *Citrobacter freundii* B38**

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15 **Running title:** Genome of *bla*_{IMP-4}-carrying *Citrobacter freundii*

16 **Key words:** *Citrobacter freundii*, IMP-4, Tn402-like, class 1 integron, SMRT whole

17 genome sequencing, plasmid

18

19

20 **ABSTRACT**

21 Sequencing of the *bla*_{IMP-4}-carrying *C. freundii* B38 using PacBio SMRT technique
22 revealed that the genome contained a chromosome of 5,134,500 bp, and three plasmids,
23 pOZ172 (127,005 bp), pOZ181 (277,592 bp), and pOZ182 (18,467 bp). Plasmid pOZ172
24 was identified as IncFIIY, like pP10164-NDM and pNDM-EcGN174. It carries a class 1
25 integron with four cassettes: *bla*_{IMP-4}-*qacG2-aacA4-aphA15*, and a complete hybrid *tni*
26 module (*tniR-tniQ-tniB-tniA*). The recombination of *tniR* from Tn402 (identical) with
27 *tniQBA* (99%) from Tn5053 occurred within the *res* site of Tn402/5053. The Tn402/5053-
28 like integron, named Tn6017, was inserted into Tn1722 at the *res* II site. The replication,
29 partitioning and transfer systems of pOZ181 were similar to IncHI2 (e.g. R478) and
30 contained a *sul1*-type class 1 integron with the cassette array: *orf-dfrA1-orf-gcu37-aadA5*
31 linked to an upstream Tn1696 *tnpA-tnpR* and to a downstream 3'CS and ISCR1. A Tn2
32 transposon with a *bla*_{TEM-1b} β -lactamase was identified on pOZ182. Other interesting
33 resistance determinants on the B38 chromosome included MDR efflux pumps, AmpC β -
34 lactamase, and resistances to Cu, Ag, As, and Zn. This is the first report of a complete *tni*
35 module linked to a *bla*_{IMP-4} carrying class 1 integron, and together with other recently
36 reported non-*sul1* integrons, represents the emergence of a distinct evolutionary lineage
37 of class 1 integrons lacking a 3'-CS (*qacE Δ 1-sul1*). The unique cassette array, complete
38 *tni* module of Tn6017, and incompatibility group of pOZ172 suggests a different *bla*_{IMP-4}
39 evolutionary pathway in *C. freundii* B38 compared to other *bla*_{IMP-4} found in Gram-negative
40 bacteria in the Western Pacific Region.

41

42 INTRODUCTION

43 Since the 1990s there have been increasing reports of class B metallo- β -lactamase-
44 producing Gram-negative bacteria that confer resistance to carbapenems, usually
45 encoded by *bla*_{IMP/VIM/GIM/SIM/NDM} genes. These genes (except for *bla*_{NDM}) have been found
46 to be carried by class 1 integrons, except for some *bla*_{IMP-1} reported in *S. marcescens*
47 from Japan, which were carried by class 3 integrons (1) (AB070224), (2) (AF416297).
48 Most class 1 integrons containing *bla*_{IMP/VIM/GIM/SIM} (but not *bla*_{NDM}) are *su1*-type,
49 containing a 3'CS downstream. However, *bla*_{IMP-9}, *bla*_{VIM-2} and *bla*_{IMP-34} have recently
50 been found on *tniABQR*-type class 1 integrons (3-6).

51
52 The IMP-4 metallo- β -lactamase was first found in *Acinetobacter* spp. that caused
53 outbreaks in the ICU wards of a university hospital in Hong Kong (7-8) and in *C. youngae*
54 (now identified as *C. freundii* in this study) from a patient with a leg ulcer in Guangzhou,
55 China (9). Now *bla*_{IMP-4}-mediated carbapenem resistance has spread to many parts of the
56 world, particularly in Australia where it has caused serious nosocomial outbreaks by
57 different Gram-negative bacteria and appeared in various genetic contexts. The most
58 prevalent context was a Sydney multiresistance region (MRR) flanked by IS26 that
59 contains a class 1 integron carrying resistance cassettes *bla*_{IMP-4}-*qacG2*-*aacA4*-*catB3*
60 (10). Interestingly, this is the same cassette array first described in a *bla*_{IMP-4}-carrying
61 class 1 integron in *Acinetobacter* spp. from Hong Kong (8) and from Singapore (11) The
62 determinant *bla*_{IMP-4} has been reported to be found in a wide range of Gram negative
63 species and be the cause of a series of nosocomial outbreaks in the Western Pacific
64 Region. The *bla*_{IMP-4}-carrying class 1 integron on plasmid pOZ172 (156 kb) in *C. freundii*

65 B38 and its *E. coli* transconjugant B38T, first described by Hawkey *et al.* in 2001 (9), was
66 further characterized as carrying a class 1 integron with a slightly different cassette array:
67 *bla*_{IMP-4}-*qacG2*-*aacA4*-*aphA15* and a hybridTn402-like *tniABQR* module by Xiong *et al.*
68 (12). A second, *sul1*-type integron, was identified in B38 but not the transconjugant.
69 Therefore, the route of acquisition and the mode of *bla*_{IMP-4} carbapenem resistance
70 transmission in *C. freundii* B38 might differ from those reported from other *bla*_{IMP-4}
71 producers.

72 After 454 and Illumina sequencing failed to resolve the two integrons and assemble
73 their respective plasmids, we used the (SMRT) sequencing method (Pacific Biosciences,
74 USA) to analyse the whole genome of *bla*_{IMP-4}-carrying *Citrobacter freundii* B38 isolated
75 from a Guangzhou multicenter surveillance program in order to understand the
76 acquisition, evolution and dissemination of the carbapenem determinant and its
77 associated mobile elements.

79 MATERIALS AND METHODS

80 **Bacterial strain.** The *bla*_{IMP-4}-carrying *C. freundii* B38 is a clinical isolate recovered
81 during a Guangzhou (multicenter) antibiotic resistance surveillance program (GARSP,
82 1998-2001). The *bla*_{IMP-4} carrying integron was on a plasmid previously estimated as 156
83 kb, that was transferable by conjugation into *E. coli* UB1637/R (9). The antibiotic
84 susceptibilities of *C. freundii* B38 are shown in Table 1. The strain had previously been
85 identified as *C. youngae* (9), but was identified in this study as *C. freundii* with a
86 probability of 93% and biochemical profile 4405615565520211 using a VITEK® 2

87 (BioMerieux, Montreal, Canada) and 99.9% using a VITEK-MS-MALDI (BioMerieux,
88 Montreal, Canada).

89 **DNA sequencing method.** Total DNA was extracted from a culture of the
90 bacterium grown overnight in LB broth at 37°C using the Qiagen Genomic-tip 20/G kit
91 (Qiagen, Toronto, Canada) and quantified by using a fluorometric, picogreen-based
92 method as well as on an agarose gel to confirm the quality and high molecular weight of
93 the isolated DNA. The genome was sequenced by Single Molecule Real Time (SMRT)
94 technique using a PacBio platform (Pacific Biosciences, Menlo Park, CA) at McGill
95 University Genome Québec Innovation Centre.

96 **Genome assembly and analysis.** The genome was assembled *de novo* using the
97 hierarchical genome-assembly process (HGAP) and proofread in PacBio (13-14). Further
98 editing and manual annotation were carried out by using RAST (15-16), Prodigal (17),
99 GCG (version 11.1; Accelrys Inc., San Diego, CA), CGView (18) and Artemis (release
100 13.2.0) (19).

101 **Nucleotide sequence accession numbers.** The complete sequence of the
102 chromosome has been submitted to GenBank under accession number CP016762.
103 Plasmids pOZ172, pOZ181 and pOZ182 were submitted under accession numbers
104 CP016763, CP016764, and CP016765, respectively.

105

106 RESULTS

107 **Overview of the genome.** The clinical isolate of *C. freundii* B38 had a chromosome
108 of 5,134,500 bp and three plasmids: pOZ172 (127,005 bp), pOZ181 (277,592 bp), and

pOZ182 (18,467 bp) assembled as intact circular molecules from SMRT sequencing. The largest plasmid, pOZ181, was only identified using SMRT sequencing and was not recovered in the previous study (9), due to limitations of the rapid plasmid isolation method used (20). The *bla*_{IMP-4}-carrying plasmid pOZ172 was 127 kb in size. The chromosome had a GC content of 51.7%, with a total of 4905 open reading frames identified by Prodigal including 23 pseudogenes. The strain was phenotypically identified as *C. freundii*. Whole genome sequencing (WGS) revealed B38 to be closest to *C. freundii* strains RLS1, CAV1741 and CAV1321, and more distant from CFNIH1 and P10159. The genome map is shown in Fig. S1 and key features of the *C. freundii* B38 genome are listed in Table 2. The genome contains at least 3 prophages not found in other *C. freundii* strains and 15 genomic islands with 10 or more genes and unique to B38. Among these are islands with genes for tellurite resistance and for D-tagatose metabolism (used to differentiate among *Citrobacter* species). Resistance genes identified on the chromosome included genes for β -lactamases, efflux pumps, a MAR locus and resistance to heavy metals (copper, silver, and arsenic).

IncF-like plasmid pOZ172

General features of the pOZ172 sequence. Plasmid pOZ172 contains 127 predicted coding regions including 4 pseudogenes, with 18 percent (23/127) of the open reading frames (ORFs) encoding hypothetical proteins, as identified by Prodigal and manually annotated with Artemis. The replication, partitioning and transfer systems showed similarity to other sequenced plasmids in GenBank. A 50 kb block of the plasmid sequence (bp 10663 to 62387), including the replication and transfer region, showed the most similarity (99%) to plasmid pP10164-NDM in *Leclercia adecarboxylata* strain P10164

132 (21), pNDM-Ec1GN574 and pNDM1_EC14653 of *Enterobacter cloacae* (22-23),
133 pRJF866 of *Klebsiella pneumonia* RJF866 (KF732966, unpublished) and pKOX_NDM1
134 of *Klebsiella oxytoca* E718 (24). The plasmid map of pOZ172 shows the genes and their
135 locations (Fig. 1). The IncFIIY maintenance, replication, and transfer modules of this
136 plasmid, homologous to those of these plasmids, are indicated in Fig. 1. The IncFII
137 plasmid replication initiator proteins RepA and RepB are 99% identical to those of
138 pRJF866 and pKOX_NDM1. The RepFIB RepA is identical to those of *K. pneumoniae*
139 KPNIH27 plasmid pKPN-262 (CP007734), strain 997 pc15-k (HQ202266), etc. (Table 3).
140 Also, three IncFII-type plasmids pKP02022 (KF719972), pKP09085 (KF719970) and
141 pKP007 (KF719971) that carry *bla*_{CTX-M-15} and isolated from three *K. pneumonia* in S.
142 Korea also contain RepFIB RepA that is identical to that found in pOZ172 (25). Plasmid
143 pOZ172 was typed as IncFIIY according to the RST scheme for IncF plasmids (26). The
144 plasmid partitioning proteins ParA and ParB are 99% and 100% identical to their
145 equivalents in plasmid II of *K. pneumoniae* strain Kp52.145 and highly similar to many
146 others (Table 3).

147

148 **Antibiotic resistance genes and their mobile elements.** The *bla*_{IMP-4}-carrying
149 class 1 integron in pOZ172 plasmid in *C. freundii* B38 contained four cassettes: *bla*_{IMP-4}-
150 *qacG2-aacA4-aphA15* and a complete but hybrid *tni* module (*tniR-tniQ-tniB-tniA*)
151 composed of *tniR* from Tn402 (identical) and *tniQ-tniB-tniA* from that of Tn5053 (6 nt
152 difference) (Fig. 2). The recombination of *tniR* from Tn402 with the *tniQBA* from Tn5053
153 occurred within the *res* site of Tn402/5053 (Fig. S4). This integron was flanked upstream
154 by the Tn1722 methyl-accepting chemotaxis protein (*mcp*) gene, and downstream by

155 Tn1722 *tnpRA*. The resistant mobile element was formed by insertion of the Tn402/5053-
156 like integron, named Tn6017, into the *res II* site of Tn1722.

157 A potential transposon contains an ABC transport system and an RND efflux
158 transporter subunit flanked by inverted repeats of IS4321R (88567-95564). Various
159 insertion sequences were identified in pOZ172 including IS*Cfr12*, IS*Sen4*, IS1, IS*Kpn26*
160 (3 copies), IS26, IS903B and ISEc36.

161 **IncHI2-like plasmid pOZ181**

162 **General features of the pOZ181 sequence.** The plasmid pOZ181 has a length of
163 277,592 bp and contains 284 predicted coding regions including 7 pseudogenes, with 45
164 percent (129/284) of the open reading frames (ORFs) encoding hypothetical proteins, as
165 identified by Prodigal and manually annotated. The key features of the plasmid, such as
166 the replication, stability and transfer systems, showed similarity to several other
167 sequenced plasmids, including the well-characterized IncHI2 plasmid R478 of *S.*
168 *marcescens* (BX664015, Table 3) (99% identity over 68% of pOZ181) (27). The plasmid
169 map of pOZ181 is shown in Fig. S2. The major features of pOZ181 are shown in Table
170 3. The dual replication and transfer modules encoded on pOZ181 are similar to those of
171 R478, containing two functional iteron-based plasmid replication determinants *repHI2A*
172 and *repH1A* (27). The plasmid replication proteins encoded by *repHI2A* and by *repH1A* of
173 pOZ181 are 99-100% identical to those of R478. The two transfer/partition regions on
174 pOZ181 are homologous to the *tra2* and *tra1* regions of R478 except for an 11 kb
175 insertion between *parMR* and *htdA* in pOZ181. Genes for the plasmid partitioning
176 proteins ParA and ParB as well as for ParM and ParR are 99% identical to those of R478
177 (Table 3).

178 **Resistance genes and their mobile elements.** The plasmid pOZ181 carries a *sul1*
179 type class 1 integron which has a 5'-CS and four cassettes: *orf-dfrA1-gcu37-aadA5*;
180 flanked upstream by a Tn1696-like *tnpR-tnpA* and downstream by 3'-CS and an *ISCR1*
181 transposase (Fig. 2). This *sul1*-type integron was not found in the transconjugant *E. coli*
182 B38T and was identified as being located on pOZ181 by SMRT sequencing. Other
183 resistance genes include: 16S rRNA methylase *armA* downstream of *ISCR1*, macrolide
184 efflux pump *msr(E)*, macrolide 2'-phosphotransferase *mph(E)*, aminoglycoside 3'-
185 phosphotransferase *aphA7*, and a glyoxalase/bleomycin resistance protein/dioxygenase.

186 The *ter* operon (ca.16.8 kb) consists of *terY3-Y2-X-Y1-W*-as well as *terZ-A-B-C-D-*
187 *E-F*. There were six intervening ORFs identified between *terW* and *terZ*. The operon is
188 highly similar (99%) to the *ter* operon in R478 (27), as well as to those of pK29 (28),
189 pENT-8a4 (CP008899) and pEC-IMP (EU855787) among others (Table 3). The arsenical
190 resistance operon consists of *arsC-B-R-H*. The *ars* operon is identical to those of R478,
191 pENT-8a4 from *Enterobacter cloacae* ECNIH3 (CP008899) and pKPC-272 from ECNIH2
192 (CP008825) as well as to those of pK29 (EF382672) from *K. pneumoniae* NK29 and
193 pSTM-A54650 from *S. typhimurium* (LK056646) (Table 3). A nickel-cobalt efflux system
194 RcnA-RcnR was also identified and was 99% identical to that of pENT-8a4 (CP008899),
195 pKPC-272 (CP008825) from *Enterobacter cloacae* ECNIH3 and ECNIH2, among many
196 others.

197 There are three copies of *IS1* and of *ISKpn26*, and three identical copies of *IS26*.
198 Two of the *IS26* copies flank the *aphA7* gene and form a potential transposon. There are
199 two copies of *IS903B*. The others include *IS4321*, *ISCR1*, *ISEc28* and *ISEc29*.

200 Plasmid pOZ182

201 This smallest plasmid in *C. freundii* B38 is 18,467 bp in length. It has 22 open
202 reading frames of which 73% are hypothetical proteins. The plasmid backbone shows no
203 similarity to plasmid sequences in GenBank. The key features of this plasmid are that it
204 contains a Tn2 transposon and a TEM-1b β -lactamase. The genetic map of pOZ182 is
205 shown in Fig. S3.

206

207 DISCUSSION

208 The *bla*_{IMP-4} in *C. freundii* B38 was identified in the second report of transferable
209 carbapenemase genes in *Enterobacteriaceae* outside of Japan and the first report in the
210 Mainland China (9). Unlike *bla*_{IMP-9} in *P. aeruginosa*, which was described at the same
211 time and place and found to be on a narrow host range IncP2 plasmid of *Pseudomonas*
212 (3, 29), *bla*_{IMP-4} has been observed to spread to a variety of Gram-negative species,
213 plasmid incompatibility groups and genetic contexts in clinical, animal, and environmental
214 isolates in the last 15 years (10, 30-33). Other reported *bla*_{IMP-4} is most commonly
215 encoded by IncL/M and IncA/C2 plasmids (Fig. 2B), typically by the cassette array *bla*_{IMP-}
216 *4-qacG2-aacA4-catB3* in a class 1 integron (10, 34). This cassette array was described in
217 *Acinetobacter baumannii* from a Hong Kong outbreak (7-8), and from Singapore (11); *K.*
218 *pneumoniae* pIMP-PH114 from Hong Kong (35); *E. cloacae* from Australia (32), *K.*
219 *pneumoniae* pJIBE401 (10); *Enterobacter cloacae* plasmid pEI1573 (34); and
220 *Enterobacteriaceae* in silver gulls in Australia (33) where the *bla*_{IMP-4} cassette is in a *sul1*-
221 type class 1 integron (i.e. with a 3'CS). Additionally, there is a *sul1*-type class 1 integron

222 from a *K. pneumoniae* isolated from Shanghai that has a single *bla*_{IMP-4} cassette with a
223 group II intron in its *attC* site (36).

224 The context of the IMP-4 carbapenem resistance cassette in *C. freundii* B38 is
225 unique and has evolved in a different manner. First, the cassette array is *bla*_{IMP-4}-*qacG2*-
226 *aacA4-aphA15* (*aphA15* instead of *catB3*); second, it is on a *tniABQR*-type class 1
227 integron, Tn6017, on pOZ172; finally, the element is on an IncFIY plasmid (Fig. 2).

228 Tn6017 may be the product of a Tn402-like element, bearing a class 1 integron and
229 a hybrid T402/Tn5053-like *tni* module, inserted into the *res* II site of Tn1722 (Fig. S4) (37).
230 The hybrid transposon composed of *tniR* (Tn402) and *tniQBA* (Tn5053) resulted from an
231 event of site-specific recombination at the position TATACGTTC within the *res* site (Fig.
232 S4 part C). Tn5053 and Tn402 *tni* genes are known to complement each other (38). A
233 similar Tn402/5053 hybrid exists as an integron encoding *aacA4-bla*_{VIM-2} in plasmid
234 PPV2-2 of *P. putida* (39). The finding of Tn6017 together with recent reports of Tn402-
235 like *tniABQR*-type *bla*_{VIM/IMP}-carrying class 1 integrons e.g. those of pDCPR1 (4) and
236 pOZ176 (3), may represent the emergence of a distinct evolutionary lineage of class 1
237 integrons lacking a 3'CS (*qacEΔ1-sul1*)-type 3'CS and instead descended either 1)
238 directly from a Tn402-like element containing only *intI1* and *tniRQBA* (40) or 2) from such
239 an element already carrying *qacE* (41). Until the recent appearance of carbapenemase-
240 encoding *tniABQR*-type integrons, the only example of a resistance integron of this type
241 was Tn402 itself. These integrons would have escaped the detection of class 1 integrons
242 with primers 5'-CS and 3'-CS.

243 The second class 1 integron in B38 was the traditional *su1* type and is encoded by
244 pOZ181 – an IncHI2 plasmid (Fig. 2). It contains four cassettes in the order *orf-dfrA1-*
245 *gcu37-aadA5*. There are three distinct plasmids in B38, with pOZ172 carrying *bla*_{IMP-4}
246 being transferable into *E. coli* UB1637/R (9). The order of integron and cassette arrival in
247 B38, with the latter usually from right to left (due to the preference for *attI* x *attC* for
248 cassette integration) (42) may reflect the history of antibiotic selective pressure in this
249 strain, with *bla*_{IMP-4} the most recent acquisition.

250 In the past decade, genes encoding the class B metallo-carbapenemase *bla*_{IMP-4}
251 gene were also found to co-exist with those encoding a class A serine carbapenemase
252 *bla*_{KPC-2} in a *K. pneumoniae* from China (43), a class D carbapenemase *bla*_{OXA-58} in
253 *Acinetobacter* spp. in Australia and Singapore; (11, 44) and another class B metallo-
254 carbapenemase *bla*_{NDM-1} (45). The IMP-4-producing *K. pneumoniae* was also isolated
255 from three infants in a NICU in the US during the period November 2009 to June 2010,
256 and the patients had no foreign travel histories, however, the genetic contexts flanking
257 the *bla*_{IMP-4} genes in these strains were not characterized (46). The association of
258 integrons with mobile elements such as transposons and/or plasmids facilitates horizontal
259 transfer of resistances at the intra- and inter-species levels (47). Tn21, Tn1696 and their
260 relatives are important vehicles for acquisition and horizontal transfer of resistance in
261 Gram-negative bacteria (48)(49).

262 Analysis of the *C. freundii* B38 genome revealed many other antibiotic and heavy
263 metal resistance determinants besides the cassettes in the two integrons. They were
264 found not only on plasmids but also on the chromosome (Table 2). They included β -
265 lactamase *bla*_{TEM-1} conferring resistance to ampicillin; aminoglycoside resistance encoded

266 by *aphA7* and *armA*; heavy metal resistances by multiple mechanisms: Cu, Ag, As, Zn,
267 Te, Co, and Ni. Plasmids of IncHI2 and IncHI1 in *Enterobacteriaceae* and the IncP2 group
268 in *Pseudomonas* are often associated with tellurite resistance (50-52). The *ter* and *ars*
269 operons identified on pOZ181 were 99-100% identical to those of R478 and other
270 plasmids that carry carbapenem resistance genes such as *bla*_{IMP}, *bla*_{KPC} and *bla*_{VIM}
271 (Table 3).

272 Both large plasmids, pOZ181 and pOZ172, contain dual replication/transfer systems.
273 The replication, partitioning and stability, and conjugative transfer systems of the IncHI2
274 plasmid pOZ181 was highly similar to R478; the former contains a unique 11 kb insertion
275 near ParMR. The corresponding IncFIY region of pOZ172, identified by replicon
276 sequence typing (RST) (26, 53) is very similar to those of NDM-1 producing IncFIY
277 plasmids, however, the second replication protein RepFIB in pOZ172, while homologous
278 to many RepFIB from IncFII plasmids (Table 3) was only 60% similar to those of some
279 NDM-1 producers.

280 This is the first report of a *tniRQBA* module linked to *bla*_{IMP-4} carrying class 1
281 integrons on an IncF plasmid. Together with other recent findings of Tn402-like *tniR*
282 associated with *bla*_{VIM-2}-carrying class 1 integrons, they may represent the emergence of
283 a distinct evolutionary lineage of class 1 integrons lacking the usual *qacEΔ1-sul1* 3'CS
284 and instead descended directly from a Tn402-like element containing only *intI1* and
285 *tniRQBA*. The unique cassette array linked to a complete *tni* module in Tn6017 encoded
286 by IncF pOZ172 suggests a different *bla*_{IMP-4} evolution route in *C. freundii* B38 than other
287 *bla*_{IMP-4} found in Gram-negative bacteria in Western Pacific Region. The co-existence of
288 multiple mobile elements including the IncH and IncF conjugative plasmids with dual

289 replication systems reflects the active horizontal genetic transfer that is taking place. The
290 closed chromosome and plasmid genomes obtained in this study using PacBio
291 technology allows for a better understanding of the relationships among resistance genes,
292 mobile elements and whole plasmids.

293

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300

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480 **FIGURE LEGENDS**

481 **FIG 1.** Map of plasmid pOZ172 from *C. freundii* B38. The scale is indicated on the
482 innermost circle. The second circle is G+C skew in green (+) and purple (-), and circle 3
483 shows G+C content (deviation from the average) in black (+, outward and -, inward). The
484 next two circles illustrate positions of CDSs in minus (circle 4) and plus (circle 5) strands
485 in dark blue. The two green arcs represent two replication/transfer regions most similar
486 (99%) to the IncF plasmids in Table 3, The *bla*_{IMP-4}-carrying integron Tn6017 (in red) is
487 inserted into Tn1722 (in yellow).

488 **FIG 2.** Class 1 integrons identified on pOZ172 and pOZ181 in *C. freundii* B38 and related
489 plasmids. (A) Tn6017, a Tn402/5053-like integron, was identified on pOZ172 and was
490 inserted into the *res* II site of Tn1722, splitting the *res* site into two ½ *res*. Arrow boxes
491 show the genes and their orientations; each solid black oval indicates *attI* and each white
492 oval represents the *attC* of the preceding gene; delta (Δ) represents disrupted genes.
493 *mcp* is the gene for the methyl-acceptor protein of Tn1722. (B): Two most representative
494 *sul1*-type *bla*_{IMP-4} class 1 integrons found in GenBank. Their cassette array is the same
495 but differs from that of Tn6017. The black rectangle is 25-nt repeat IRI; the short vertical
496 lines are the 12-nt repeats of IS26. (C) A second, *sul1*-type integron was identified on
497 pOZ181 and was linked upstream to Tn1696 and downstream to an ISCR1. The small
498 rectangular white boxes represent the *res* sites adjacent to *tnpR* and the solid black
499 boxes represent the 25-bp IRI (Tn402) and IRI (Tn5053) sites; the larger solid black
500 boxes represent the 38-bp IRL and IRRI of Tn1722, as well as IRI of Tn1696; the small
501 arrows represent the direction of promoters (P and P1).

502

503 **TABLE 1.** Susceptibilities (MICs (mgL⁻¹) of *C. freundii* B38

Strain	Test	IMP	MEM	CAZ	CTX	CRO	FEP	TIM	CFP	TZP	CIP	AMK	GEN
	date												
<i>C. freundii</i>	1999.2 ^a	24	ND	256	256	256	256	256	256	32	32	256	256
B38	2000.5 ^b	0.5	6	256	256	256	256	256	256	32	32	256	256
	2002.5 ^c	2	0.5	256	256	256	ND	ND	ND	32	32	256	ND

504 a: tested in Guangzhou with Etest gradient method; b: tested in Sweden with Etest
 505 gradient method; c: tested in Birmingham with agar dilution method. IMP, imipenem;
 506 MEM, meropenem; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP,
 507 cefepime; TIM, ticarcillin/clavulanic acid; CFP, cefoperazone/sulbactam; TZP, piperacillin/
 508 tazobactam; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin. ND, not determined.

509

510

511 **TABLE 2.** The overall features of the *C. freundii* B38 genome

	Chromosome	pHRB381	pOZ172	pHRB382
Size (bp)	5,134,500	277,592	127,005	18,467
G+C%*	51.7	45.8	54.4	57.7
Predicted CDS	4905	277	127	22
Resistance determinants	<i>mdtABCD</i> ; MAR; heavy metals: Cu, Ag, As, Zn, Efflux pumps	heavy metals: <i>ars</i> operon; <i>ter</i> operon; <i>rcnA</i> operon antibiotics: <i>dfrA1</i> ; <i>aadA5</i> ; <i>qacEΔ1</i> ; <i>sul1</i> ; <i>armA</i> ; <i>mph(E)</i> ; <i>aphA7</i> ; <i>msr(E)</i> ; bleomycin ^R	<i>bla</i> _{IMP-4} ; <i>qacG</i> ; <i>aacA4</i> , <i>aphA15</i>	<i>bla</i> _{TEM-1}

512 *Mobile elements and insertions were not excluded.

513

514 **TABLE 3.** Comparative analysis of key features of the plasmids in *C. freundii* B38

515

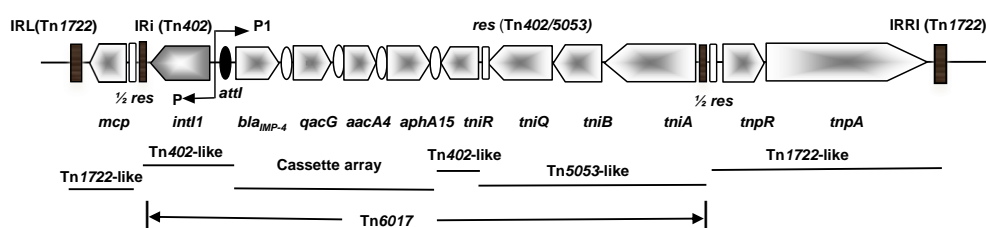
Function	Genomic(coordinates)	Homologs in GenBank
pOZ172		
Replication Transfer	<i>repAB</i> (bp: 11218-12648, c); <i>tra</i> operon (bp:14277-45250)	most similar (99%) to: pNDM-Ec1GN574, pNDM1_EC14653 (<i>E. cloacae</i>); pKOX_NDM1 (<i>K. oxytoca</i>); pP10164-NDM (<i>Leclercia adecarboxylata</i>); pYDC644, pRJF866 (<i>K. pneumonia</i>)
Replication Partition	<i>repFIB</i> (bp: 81538-82548); <i>parBA</i> (bp:78650–80787, c)	most similar (99%) to: pCAV1344-250, pKPN-262, pKPN3 ,pKP007 plasmid II str. Kp52.145, pc15-K, pUUH2392 pKP02022, pKP09085 (<i>K. pneumoniae</i>); pOU7519 (<i>S. enterica</i>); etc.
pHRB381		
Replication Transfer Partition	<i>repHI2A</i> (bp:75780-76868,c); <i>repH1A</i> (bp:90277-91152); <i>tra2</i> (bp:24119-41412); <i>tra1</i> (bp:92763-141366); <i>parAB</i> (107267-108722); <i>parMR</i> (112426-114859)	most similar (99-100%) to: R478* (<i>S. marcesens</i>); pK29 (<i>K. pneumoniae</i>); pCAV1151-296 (<i>Kluyvera intermedia</i>); pEN-08e, pENT-8a4, pMRVIM0813, pKPC- 272, pEC-IMPQ and pEC-IMP (<i>E. cloacae</i>); pSTm-A54650 (<i>S. enterica</i>), etc.
Heavy metal resistance	<i>ter</i> operon(bp:166156-182942); <i>ars</i> operon (bp:53-2937); <i>rcnA</i> operon (bp:263574- 265084)	*pHRB381 has an 11 kb insertion of hypothetical proteins between <i>parMR</i> and <i>htdA</i> of R478.
Sulphate permease	<i>sfpAB</i> operon (bp:275261- 277585)	

516

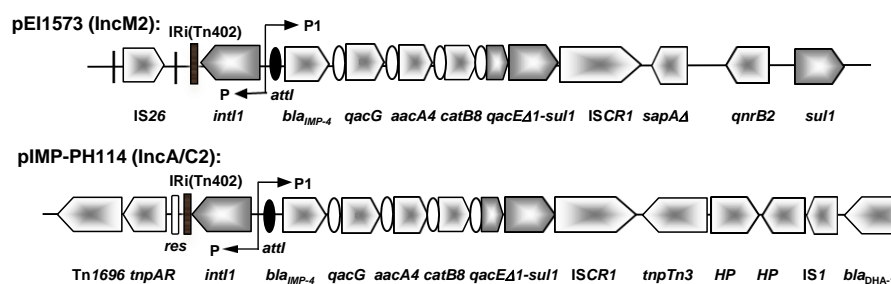
517



(A): Tn6017 in a Tn1721-like element in pOZ172 (IncFIIy):



(B): Two *sul1*-type *bla*_{IMP-4} integrons in GenBank: pEI1573 (JX101693) & pIMP-PH114 (KF250428)



(C): *sul1*-type integron in a partial Tn1696 in pHRB381(IncHI2):

